

Importance of eIF2 α Phosphorylation and Stress Granule Assembly in Alphavirus Translation Regulation

Gerald M. McNerney,* Nancy L. Kedersha,[†] Randal J. Kaufman,[‡] Paul Anderson,[†] and Peter Liljeström*[§]

*Microbiology and Tumour Biology Centre, Karolinska Institute, Stockholm 171 77, Sweden; [†]Division of Rheumatology and Immunology, Brigham and Women's Hospital, Boston, MA 02115; [‡]Howard Hughes Medical Institute, Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109-0659; and [§]Department of Vaccine Research, Swedish Institute for Infectious Disease Control, Stockholm 105 21, Sweden

Submitted February 14, 2005; Revised May 16, 2005; Accepted May 19, 2005
Monitoring Editor: Marvin P. Wickens

Alphavirus infection results in the shutoff of host protein synthesis in favor of viral translation. Here, we show that during Semliki Forest virus (SFV) infection, the translation inhibition is largely due to the activation of the cellular stress response via phosphorylation of eukaryotic translation initiation factor 2 α subunit (eIF2 α). Infection of mouse embryo fibroblasts (MEFs) expressing a nonphosphorylatable mutant of eIF2 α does not result in efficient shutoff, despite efficient viral protein production. Furthermore, we show that the SFV translation enhancer element counteracts the translation inhibition imposed by eIF2 α phosphorylation. In wild-type MEFs, viral infection induces the transient formation of stress granules (SGs) containing the cellular TIA-1/R proteins. These SGs are disassembled in the vicinity of viral RNA replication, synchronously with the switch from cellular to viral gene expression. We propose that phosphorylation of eIF2 α and the consequent SG assembly is important for shutoff to occur and that the localized SG disassembly and the presence of the enhancer aid the SFV mRNAs to elude general translational arrest.

INTRODUCTION

A hallmark of infection with many lytic viruses is the shutoff of host cell protein synthesis. Many viruses commandeer the cellular translational apparatus to increase the yield of viral protein and dampen cellular innate immune signaling. However, in some cases, this translational inhibition can itself be seen as a cellular defense mechanism, to which viruses have evolved certain levels of tolerance. An example of this is the activation of the cellular double-stranded RNA-activated protein kinase R (PKR) by many viral infections and the multifarious viral countermeasures (Gale *et al.*, 2000; Kaufman, 2000). PKR, activated by dimerization and autophosphorylation, phosphorylates the eukaryotic translation initiation factor 2 α subunit (eIF2 α). Phospho-eIF2 α restricts the activity of the guanine nuclear exchange factor eIF2B, which is responsible for the formation of the GTP-eIF2-tRNAi^{Met} ternary complex. Because translation of most cellular and viral mRNA proceeds via ternary complex-dependent mechanisms, this leads to a global decrease in protein synthesis.

Semliki Forest virus (SFV) is an RNA virus of the *Alphavirus* genus, family *Togaviridae*. The viral particles are enveloped and contain a single-stranded positive-sense 42S RNA genome. The 5' two-thirds of the capped and polyadenylated genome codes for the four viral nonstructural pro-

teins (ns_{p1-4}), whereas the structural protein-coding regions are carried on a subgenomic 26S mRNA, transcribed from the 3' one-third of the 42S RNA. Alphavirus infection of permissive cells causes shutoff between 2 and 4 h postinfection (hpi), the only proteins efficiently produced thereafter being virus encoded, expressed from the 26S mRNA. This property has contributed to the widespread use of recombinant alphaviruses as expression vectors (Xiong *et al.*, 1989; Liljeström and Garoff, 1991; Pushko *et al.*, 1997). Despite much interest in applying these vectors to biotechnology, the mechanism by which this important step in the alphavirus life cycle is achieved has remained largely unknown.

Several models have been proposed for the mechanism of alphavirus-mediated shutoff (Strauss and Strauss, 1994). It was thought that the efficient replication of viral RNA (vRNA) led to high concentrations of cytoplasmic 26S mRNA molecules, which outcompete cellular mRNAs for the available translation machinery (Kääriäinen and Ranki, 1984). Furthermore, it was thought that the consequent reduction in intracellular rNTP pools could lead to shutoff (Wengler, 1980). However, the observation that 26S mRNA-defective mutants of Sindbis alphavirus (SIN) cause efficient shutoff in infected cells suggested instead that the earlier events in 42S RNA synthesis are important for this to occur (Frolov and Schlesinger, 1994a). Other functions attributed to the viral structural proteins themselves (van Steeg *et al.*, 1984; Elgizoli *et al.*, 1989) also were suggested to be the causative agents. The advent of several alphavirus-based replicon systems showed this not to be the case (Xiong *et al.*, 1989; Liljeström and Garoff, 1991; Pushko *et al.*, 1997). Each of these systems involves the use of replication-competent alphavirus RNAs that elicit efficient shutoff while lacking the

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05-02-0124>) on June 1, 2005.

Address correspondence to: Gerald M. McNerney (gerald.mcnerney@mtc.ki.se).

structural protein-coding regions. Other well studied viral systems have translation mechanisms conferring independence to certain eIFs, which are then cleaved or otherwise inactivated (Belsham and Jackson, 2000), leading to preferential vRNA translation and host shutoff. Although, the SFV 26S mRNA has been shown to have a low requirement for both eIF4E (the cap-binding protein) and eIF4B (van Steeg *et al.*, 1981), neither of these proteins is known to be inactivated in alphavirus-infected cells. Despite these uncertainties, it remains apparent that shutoff is linked with vRNA replication, because noncytopathic vectors that do not induce complete translation inhibition tend to have mutations in the replicase region, particularly nsp2 (Agapov *et al.*, 1998; Frolov *et al.*, 1999; Perri *et al.*, 2000; Frolova *et al.*, 2002). Recently, results by Frolov and coworkers (Gorchakov *et al.*, 2004) showed that shutoff in SIN-infected cells is mediated by both PKR-dependent and -independent mechanisms, as revealed by infection of wild-type (wt) and PKR^{-/-} cells with wt and nsp2 mutant recombinant SIN.

The 5' end of SFV and SIN structural protein coding regions have been shown to contain a unique genetic element known as the translation enhancer (Frolov and Schlesinger, 1994b; Sjöberg *et al.*, 1994). Recombinant viral vectors that lack the enhancer, while eliciting efficient host cell shutoff, produce approximately 10-fold less protein than those that contain the enhancer (Frolov and Schlesinger, 1994b; Sjöberg *et al.*, 1994). These regions are predicted by RNA sequence analysis to contain very stable hairpin loops composed of mainly G:C base pairs. The translational enhancement by this element was shown to occur only in infected cells (Frolov and Schlesinger, 1994b; Sjöberg and Garoff, 1996), suggesting that its presence is an adaptation to an altered translational environment therein. Although the mechanism of translational enhancement by this element is not entirely clear, it does seem to be carried out at the initiation step of translation (Frolov and Schlesinger, 1996).

General translational inhibition also is seen in cells under conditions of environmental stress, such as heat shock. The mechanism of shutoff under these conditions is better understood. During heat shock, cells shut down the expression of normal housekeeping genes to allow the selective expression of stress response factors (e.g., heat-shock proteins [HSPs]). eIF2 α becomes phosphorylated by one of a small range of kinases, leading to reduction in ternary complex levels. The RNA binding protein TIA-1 and the related protein TIAR (collectively referred to as TIA-1/R) are then included into noncanonical preinitiation complexes known as 48S* complexes (Kedersha *et al.*, 2002). Aggregation of TIA-1/R then sequesters the components of these complexes into cytoplasmic structures known as stress granules (SGs) (Kedersha *et al.*, 1999; Gilks *et al.*, 2004). In a process referred to as mRNA triage, the mRNA is believed to be stored, pending either degradation or resumption of normal translation in the absence of stress (Anderson and Kedersha, 2002). Consistent with this model, it has been shown that during heat shock, the HSP chaperone HSP70 mRNA molecules are excluded from SGs (Kedersha and Anderson, 2002) and are efficiently translated.

TIA-1/R are multifunctional proteins and in addition to their role in SG assembly, they are involved in other diverse activities, such as translational silencing (Kedersha *et al.*, 1999; Piecyk *et al.*, 2000), alternative splicing regulation (Förch *et al.*, 2000), viral replication (Iseni *et al.*, 2002; Li *et al.*, 2002), and Fas-mediated apoptosis (Taupin *et al.*, 1995). Although TIA-1^{-/-} and TIAR^{-/-} mice have been produced previously (Beck *et al.*, 1998; Piecyk *et al.*, 2000), the presence of either TIA-1/R protein in the cells is essential for viability

(Le Guiner *et al.*, 2003), illustrated by the fact that double (TIA-1^{-/-}TIAR^{-/-}) knockout mice cannot be generated (Kedersha and Anderson, unpublished results).

We were interested to study the role of this stress-induced translation inhibition mechanism in the host shutoff observed during SFV infection and possible mechanisms of viral escape. Our results suggest that the inhibition of protein production seen during conditions of environmental stress and during SFV infection is caused by similar mechanisms. The virus seems to have adapted to this response by preventing the formation of the cytosolic SGs on the 26S mRNA molecules. Furthermore, we show that the translation enhancer only functions in the presence of high levels of phospho-eIF2 α , suggesting that it is also an adaptation to this response. Many viruses have mechanisms to inhibit the cellular defense capacity of PKR by inhibiting its activation (Kaufman, 2000). This work shows that SFV also inhibits this pathway but at a point downstream of eIF2 α phosphorylation.

MATERIALS AND METHODS

Cells and Viruses

Previously described mouse embryo fibroblast (MEF) cell lines (Scheuner *et al.*, 2001; Li *et al.*, 2002) were cultured in DMEM (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma) and 2 mM L-glutamine (complete medium). In each experiment with these cells, wt controls were of the same genetic background. Stocks of wt SFV were generated from the pSP6-SFV4 infectious clone as described previously (Liljeström *et al.*, 1991). The pSFV-b7lacZ and pSFV-b7EGFP clones contain the lacZ and EGFP genes, respectively, fused to the translation enhancer sequence (Sjöberg *et al.*, 1994). Stocks of SFV1-lacZ (Liljeström and Garoff, 1991), SFV-b7lacZ, SFV1-EGFP, and SFV-b7EGFP recombinant virus particles were prepared as described previously (Smerdou and Liljeström, 1999). Titers of viruses were quantified by either plaque assay (wt) or by immunofluorescence (recombinants) on wt MEF cells. SFV-b7EGFP was UV-inactivated using Amersham Biosciences (Piscataway, NJ) UV Cross-linker at 2000 kJ/cm² for 30 s. SFV stocks were used for infection as follows: cell monolayers were washed with phosphate-buffered saline (PBS), and virus was added in 300 μ l of Glasgow minimum essential medium with 0.2% bovine serum albumin with periodic shaking for 1 h at 37°C. Virus solutions were then removed, and cells were washed with PBS before prewarmed complete medium was added. Where indicated, cells were stressed by the addition of sodium arsenite to 0.5 mM in complete medium.

Plasmids

The enhanced green fluorescent protein (EGFP) gene was cloned into *Bam*HI/*Kpn*I-cut pcDNA3.1 to give pcDNA-EGFP. For pcDNA-b12A-EGFP, a fragment encoding the SFV 26S mRNA 5'-untranslated region, the enhancer sequence and the foot-and-mouth disease virus 2A autoprotease in frame with the EGFP gene was constructed by overlap PCR and ligated into *Bam*HI/*Kpn*I-cut pcDNA3.1. Transient transfection was performed with Lipofectamine (Invitrogen, Carlsbad, CA).

Western Blotting

For analysis of protein by Western blotting, cells were lysed on ice in lysis buffer (1% NP-40, 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 2 mM EDTA), clarified by centrifugation at 6000 \times g for 5 min in a microcentrifuge at 4°C, separated by SDS-PAGE, transferred to Hybond-P (Amersham Biosciences) membranes, and blotted with rabbit polyclonal antibodies specific for either phospho-eIF2 α (phosphorylated on serine 51; BioSource International, Camarillo, CA) or total eIF2 α (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit serum (BD Biosciences, San Jose, CA). Chemiluminescence was detected using the ECL reagents (Amersham Biosciences). Membranes were stripped by washing in 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7, for 30 min at 50°C, washed, blocked, and probed again as described above.

Protein Expression Assays

To control for different cell lines growth rates in the total protein expression experiments, MEFs were seeded at equal numbers into 35-mm dishes (Falcon; BD Biosciences Discovery Labware, Bedford, MA) 4 h before the experiments. For metabolic labeling of virus-infected cultures, cells were incubated first in methionine-free minimum essential medium supplemented with 2 mM L-glutamine (starvation medium) for 20 min. Newly produced proteins were

then labeled by incubation in starvation medium supplemented with 50 $\mu\text{Ci}/\text{ml}$ [^{35}S]methionine (Amersham Biosciences) for 10 min (pulse medium). Cells were then washed in cold PBS, lysed on ice in lysis buffer, and clarified by centrifugation at $6000 \times g$ for 5 min in a microcentrifuge at 4°C . Supernatants were then analyzed on 10% SDS-PAGE gels, dried, and subjected to autoradiography. In vitro β -galactosidase (β -Gal) assays were performed as described previously (Liljeström and Garoff, 1991). For metabolic labeling of transiently transfected cultures, newly produced proteins were labeled directly by incubation in pulse medium for 1 h, with or without sodium arsenite as indicated. For direct quantitation of protein levels, EGFP was immunoprecipitated from lysates using green fluorescent protein (GFP) monoclonal antibody (BD Biosciences Clontech, Palo Alto, CA) and protein G-Sepharose (Amersham Biosciences). Unincorporated [^{35}S]methionine was removed from lysates by Microcon YM3 filtration (Millipore, Billerica, MA). Samples of each were mixed with Emulsifier-Safe (PerkinElmer Life and Analytical Sciences, Boston, MA) and quantified by scintillation counting.

Immunofluorescence

Antibodies used for immunofluorescence include goat anti-TIA-1 (Santa Cruz Biotechnology), mouse anti-TIA-1/R (3E6; Taupin *et al.*, 1995), goat anti-eIF3 (Santa Cruz Biotechnology), mouse anti-bromouridine triphosphate (Br-dUTP) (Stressgen Biotechnologies, Victoria, British Columbia, Canada), and mouse anti-digoxigenin (DIG) (Roche Diagnostics, Indianapolis, IN). Secondary antibodies were Cy2- and Cy3-conjugated donkey anti-goat sera and Cy3-conjugated donkey anti-mouse serum (all ML grade from Jackson ImmunoResearch Laboratories, West Grove, PA). Cells grown on coverslips were fixed by incubation in 4% paraformaldehyde (in PBS) for 8–10 min at room temperature followed by incubation in methanol for 8–10 min at -20°C . Coverslips were rinsed three times in PBS before incubation in blocking buffer (5% horse serum [Invitrogen], 0.02% sodium azide in PBS) for 1 h at room temperature. Primary antibodies were diluted in blocking buffer and incubated with the cells for 1–3 h. After rinsing in PBS, secondary antibodies were diluted in blocking buffer and incubated with the cells for 1–3 h. This secondary incubation contained 0.5 $\mu\text{g}/\text{ml}$ Hoechst 33258 (Molecular Probes, Eugene, OR) for identification of cell nuclei. Washed coverslips were then mounted in Vinol mounting medium, and images were captured using a Leitz DM RB fluorescent microscope with a Hamamatsu cooled charged-coupled device camera C4880. Images were processed and compiled using Adobe Photoshop software.

RNA Quantitation by [^{14}C]Uridine Labeling

Equal numbers of MEFs were infected with wt SFV at multiplicity of infection (MOI) 10 (as described above). Thirty minutes before each labeling period, 1 $\mu\text{g}/\text{ml}$ actinomycin D (ActD) (Sigma) was added to the medium to block cellular RNA synthesis. Then, 1 $\mu\text{Ci}/\text{ml}$ [^{14}C]uridine (Amersham Biosciences) was added so that all newly produced vRNA would incorporate this label. After the labeling period, total cell RNA was then isolated with TRIzol reagent (Invitrogen) according to the manufacturer's instructions and analyzed by agarose gel electrophoresis. The gel was then soaked for 30 min in 1 M salicylic acid (Sigma), dried, and exposed to x-ray film (Fujifilm, Tokyo, Japan).

Labeling of Newly Produced vRNA with BrUTP

For labeling of newly synthesized vRNA, MEFs grown on 13-mm-diameter glass coverslips, were infected with wt SFV at low MOI. Thirty minutes before and throughout each labeling period, ActD was added to the growth medium to 1 $\mu\text{g}/\text{ml}$. Lipofectamine (Invitrogen) was used to facilitate entry of the BrUTP label into the cells, according to the manufacturer's instructions. Briefly, growth medium was removed and after three PBS washes, it was replaced with Opti-MEM (Invitrogen) containing the appropriate volumes of Lipofectamine reagent and ActD and 10 mM BrUTP (Sigma). After the labeling period, cells were washed three times in PBS and fixed and processed for immunofluorescence as described above.

In Situ Hybridization (ISH)

The PCR DIG probe synthesis kit (Roche Diagnostics) was used to generate a virus-specific double-stranded DNA (dsDNA) probe labeled with DIG according to the manufacturer's instructions. The template for the PCR reaction was pSFVHelper1 (Liljeström and Garoff, 1991) and the primers were 5'-TATGCTCCCGGGCGACGAGCTGCAGT-3' (forward primer) and 5'-GTACACCCCGGGGTAACCTTGCA-3' (reverse), amplifying a 506-base pair region of the viral structural protein-coding region. ISH analysis for total vRNA (42S + 26S) was performed as follows. After fixing (as for immunofluorescence), cells were washed twice in $2\times$ SSC. DIG containing dsDNA probes (denatured by boiling and diluted in hybridization buffer [0.5% formamide, $4\times$ SSC, $1\times$ Denhardt's reagent, 20 mM sodium phosphate, pH 7.0, 5% (wt/vol) dextran sulfate, 200 mM dithiothreitol, and 0.5 mg/ml denatured salmon sperm DNA] were hybridized to the cells at 44°C overnight in a humid chamber. Cells were then washed with $2\times$ SSC and incubated with primary antibodies (typically goat anti-TIA-1 and mouse anti-DIG) in $4\times$ SSC containing 0.1% NP-40 for 1 h at room temperature. After washing in $4\times$ SSC,

cells were incubated with the requisite secondary antibodies and 0.5 $\mu\text{g}/\text{ml}$ Hoechst 33258 (Molecular Probes) in $2\times$ SSC containing 0.2% NP-40 for 1 h at room temperature. Cells were then washed sequentially in $4\times$ SSC and $2\times$ SSC and mounted and analyzed as for immunofluorescence. Controls performed with each experiment included mock-infected cells, mock-hybridized infected cells, and infected cells hybridized with non-DIG-containing probes. Furthermore, low MOI was used for easy comparison of background staining from noninfected cells.

RESULTS

Phosphorylation of eIF2 α on Serine 51 Is Necessary for Shutoff in SFV-infected Cells

To determine whether the cellular stress response is involved in translation regulation during SFV infection, we studied translation in SV129 MEFs expressing a mutant form of eIF2 α (Ser51Ala or A/A) that cannot be phosphorylated on serine 51 (Scheuner *et al.*, 2001). Equal numbers of mutant and wt cells were infected with wt SFV at MOI 10. At different time points after infection, total protein production was analyzed by metabolic labeling. Figure 1A shows that in SV129 wt MEFs (S/S), efficient host cell translation inhibition was observed after ~ 3 h of infection. Thereafter, newly produced proteins were predominantly viral, although a small residual level of cellular protein synthesis could be detected, probably from uninfected cells in the culture. In the MEFs expressing the nonphosphorylatable mutant (A/A), host shutoff did not occur at such efficiency, although the earliest production of viral proteins was not delayed relative to the S/S cells. Both sets of cells were infected at similar levels as determined by immunofluorescence (our unpublished data).

Western blotting was used to confirm the eIF2 α phosphorylation status in these cells. Again, equal numbers of S/S and A/A cells were infected, and lysates were taken for analysis at 5 hpi. Figure 1B, top, shows that eIF2 α was phosphorylated after infection with SFV but only in the S/S cells, as expected. Figure 1B, bottom, shows the same membrane that was stripped and reprobed for total eIF2 α , showing that eIF2 α protein expression was not altered in the mutant cells. Together, these results show that eIF2 α becomes phosphorylated during infection with SFV and that this phosphorylation is important for shutoff of host mRNA translation.

The SFV Translational Enhancer Counteracts the Translation Block Imposed by eIF2 α Phosphorylation

To explain the efficient translation of viral mRNAs in the presence of eIF2 α phosphorylation, we initially studied the effect of the viral translational enhancer element in the S/S and A/A cell lines. As discussed, the enhancement of this element has been previously shown only to occur in infected cells and that it most likely functions at the initiation stage of translation. Analysis of protein production levels from vectors containing the enhancer in the A/A mutant cells therefore would indicate the relationship between the enhancer and eIF2 α phosphorylation. Equal numbers of S/S and A/A cells were infected at MOI 10 with either SFV-lacZ or SFV-b7lacZ, lacking or containing the enhancer, respectively. In previous work, the replication of similar recombinant SFV genomes led to shutoff as efficiently as wt SFV (Liljeström and Garoff, 1991). Equal infection of each cell line in this experiment was verified by an in situ β -Gal assay (our unpublished data). Lysates were taken at different times postinfection and analyzed for β -gal protein levels by an in vitro assay. As expected, infection with the enhancer-containing vector led to greatly increased β -Gal levels in wt (S/S) MEFs (Figure 2A, left). 12-fold more β -Gal protein was

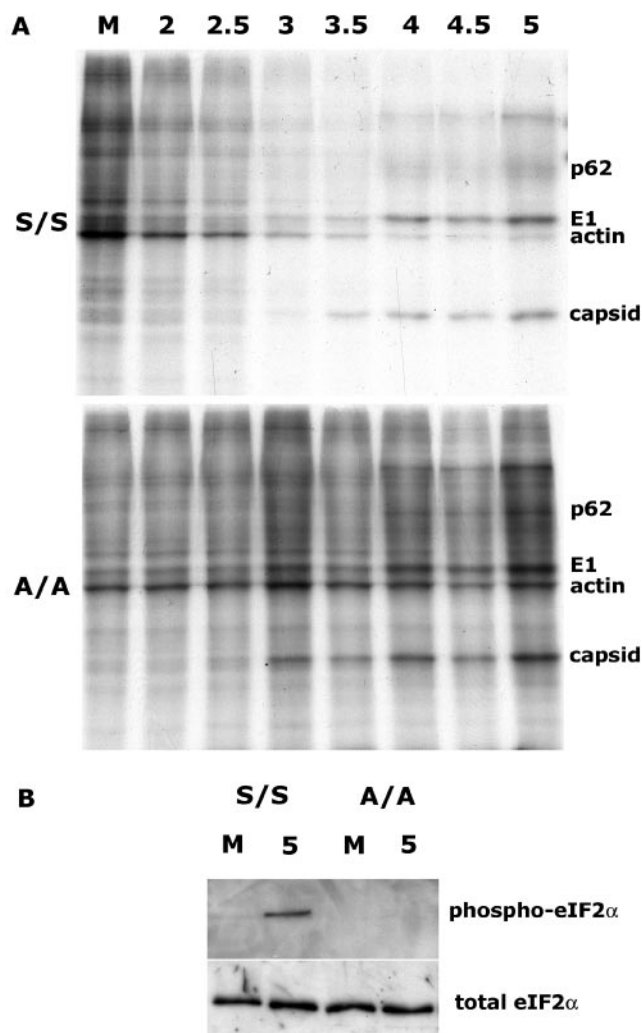


Figure 1. Phosphorylation of eIF2 α is necessary for efficient shutoff to occur in SFV-infected cells. (A) Total protein production at various times in infected wt eIF2 α (S/S) and nonphosphorylatable mutant eIF2 α (A/A)-expressing MEF cells was analyzed as indicated in *Materials and Methods*. Positions of the cellular protein actin and viral proteins p62, E1, and capsid are indicated. All lanes are labeled in hpi or as M, mock-infected cells. (B) The phosphorylation state of eIF2 α in these cells 5 h postinfection or mock infection was determined by Western blotting for phospho-eIF2 α (top) and related to total eIF2 α levels by stripping and reprobing the membrane (bottom).

detected in the S/S cells after infection with SFV-b7lacZ than after infection with SFV-lacZ, an increase consistent with other reports of alphavirus enhancer activity in infected wt cells (Frolov and Schlesinger, 1994b; Sjöberg *et al.*, 1994). Interestingly, in the A/A mutant cells, both recombinant viruses expressed high levels of β -Gal with SFV-lacZ (lacking the enhancer), producing $\sim 50\%$ more protein than the enhancer-containing virus (Figure 2B, right). Thus, the enhancer is necessary for efficient viral protein synthesis but only in the presence of eIF2 α phosphorylation.

In an alternative approach to study the relationship between the enhancer activity and eIF2 α phosphorylation, we constructed plasmids for transient transfection of the EGFP gene, either alone (pcDNA-EGFP) or in fusion with the enhancer sequence (pcDNA-b12A-EGFP). We then assayed

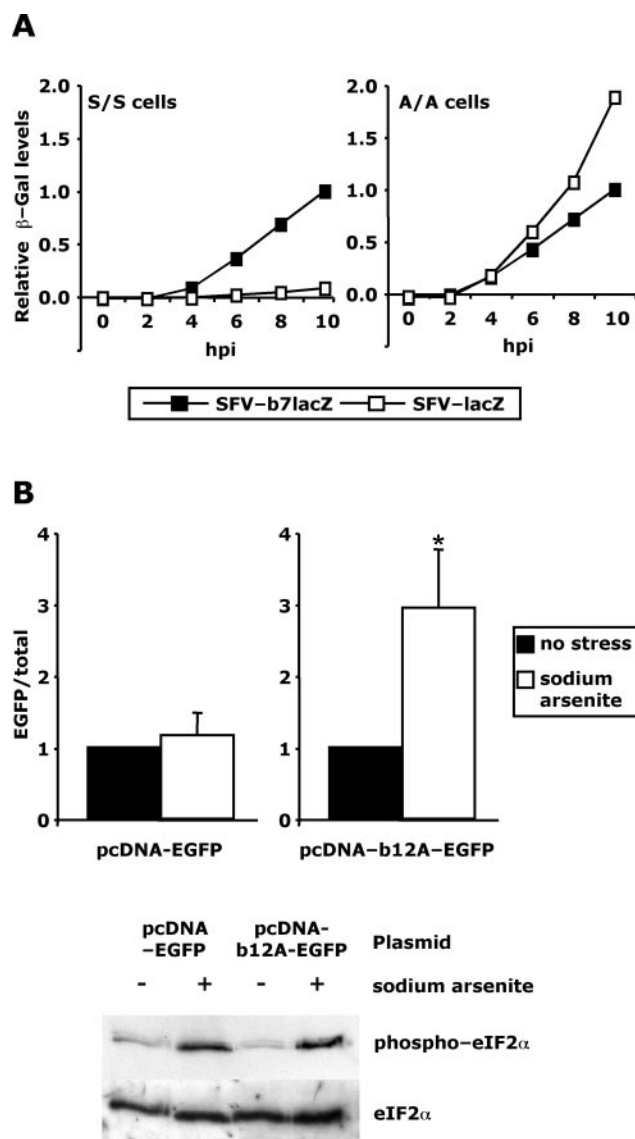


Figure 2. The SFV translational enhancer counteracts the translation block imposed by eIF2 α phosphorylation. (A) Equal numbers of S/S and A/A cells were infected with SFV-b7lacZ or SFV-lacZ at MOI 10. Lysates were taken at different times postinfection and analyzed by an *in vitro* β -Gal assay. For each cell line, all protein levels are presented relative to the level of protein present in cells infected for 10 h with enhancer-containing virus. In this way, the level of translational enhancement in the cells can easily be appreciated. The results are averages of three independent experiments with similar results. (B) BHK cells were transfected with either pcDNA-EGFP or pcDNA-b12A-EGFP. Twenty-four hours later, the cells were labeled with [35 S]methionine for 1 h, in the presence or absence of sodium arsenite. EGFP levels were determined by immunoprecipitation and scintillation counting. Total protein levels were determined by scintillation counting after removal of unincorporated label. For each construct, the results are presented as EGFP/total and shown relative to that value for nonstressed cells. Thus, the efficiency of each transfected gene can be related to total cellular translation in each condition. The results are averages of four independent experiments with similar results. Error bars are SD, and * indicates $p < 0.05$ versus nonstressed cells (Student's *t* test). Shown in the lower panel is Western blot analysis for phospho- and total eIF2 α in each set of cells.

protein expression levels in the context of sodium arsenite-induced eIF2 α phosphorylation. Baby hamster kidney (BHK) cells were transfected with either plasmid and at 24 h, the cells were metabolically labeled with [³⁵S]methionine in the presence or absence of arsenite. A measure of the translational efficiency, under each condition, of the plasmid-derived transcripts relative to cellular transcripts was determined and related to that in the absence of stress for each plasmid. Although the levels of total protein expression were reduced ~10-fold in all cells under stress, the relative efficiencies of the plasmid-derived mRNAs differed (Figure 2B). The relative expression of pcDNA-EGFP mRNA was not altered by stress treatment (left), and thus we conclude that it was inhibited by factor similar to that of the cellular mRNAs. In contrast however, it can be seen that the relative expression of the pcDNA-b12A-EGFP-derived mRNA was increased threefold in the presence of arsenite (right). Figure 2B, bottom, shows that the arsenite treatment in each case led to similar levels of eIF2 α phosphorylation. The results in Figure 2 together demonstrate that the presence of the enhancer on the 26S mRNA allows the RNA to be efficiently translated in the absence of normal ternary complex levels as observed during infection or stress induced by other means.

Transient Assembly of SGs in SFV-infected Cells

Because it has been shown that the formation of SGs is a mechanism to down-regulate cellular housekeeping translation during stress and that phosphorylation of eIF2 α is sufficient for their formation (Kedersha *et al.*, 1999), we determined whether these structures were indeed formed during SFV infection. Accordingly, we analyzed the localization of TIA-1/R proteins in SFV-infected cells by immunofluorescence. Figure 3A shows a time-course experiment in which MEFs were infected at MOI 1 with SFV-b7EGFP, fixed at various time points, and stained for TIA-1. Low MOI was used so that noninfected cells would be visible in each field, serving as internal controls. Shortly after infection, TIA-1 completely relocated to the cytoplasm and was found in SGs. This change in localization corresponded in time to the change in protein production from host to viral proteins, as evidenced by the EGFP production visible in the same cells. As the infection proceeded, however, there was a marked inverse relationship between the intensity of EGFP signal and the number of SGs in each cell. This was evident in all cells viewed at 4 hpi or later. At the conclusion of these time-course experiments (8 hpi), all infected cells showed very high viral protein production (EGFP) and complete TIA-1 relocation out of SGs, into a diffuse distribution throughout the cytoplasm. Cells that were mock infected or infected with UV-inactivated SFV-b7EGFP did not show alteration of TIA-1 localization after 8 h, demonstrating the requirement for vRNA replication for this to occur. Similar results were seen using 3E6, an antibody predominantly directed to TIAR (our unpublished data).

Confirming that this transient granular TIA-1/R staining does indeed represent SGs, we also observed eIF3 (a component of the translation preinitiation complex and another SG marker; Kedersha *et al.*, 2002) colocalization with TIA-1 in SGs in cells infected at MOI 1 with wt SFV (Figure 3B). Furthermore, SFV-induced SGs were in dynamic equilibrium with polysomes as shown by their differential reactions to treatments with puromycin or cycloheximide (our unpublished data) as observed previously for arsenite-induced SGs (Kedersha *et al.*, 2000). These experiments suggest that SG formation during SFV infection is transient and occurs at the time of host shutoff, i.e., when the profile of protein production changes from cellular to viral.

Because SG components are in dynamic equilibrium with polysomes (Kedersha *et al.*, 2000), we investigated whether the SG disassembly seen at later times (Figure 3A) was a result of the efficient translation of the viral 26S mRNA competing with the SGs for the 48S complexes. Accordingly, we infected MEFs with SFV1-EGFP, which does not contain the translation enhancer element and thus does not direct as efficient translation of the 26S mRNA as wt SFV or SFV-b7EGFP, and analyzed the levels of SG assembly and disassembly (Figure 3C). Despite the very low level of translation of the 26S mRNA of this virus at these early times, the complete disassembly of SGs was observed at similar times for this virus as for the enhancer-containing viruses.

Protein Production during the Early Stages of SFV Infection of TIA-1^{-/-} Cell Lines

We wanted to determine the rate and extent of shutoff in the absence of the TIA-1/R proteins. However, because total knockout of both TIA-1/R proteins is lethal (Le Guiner *et al.*, 2003), it is technically impossible to assay SFV-induced effects in their absence. Thus, the TIA-1^{-/-} MEF cells (Piecny *et al.*, 2000) constitute a significant knockdown (rather than knockout) of total TIA-1/R protein, and results regarding SFV-induced shutoff and SG formation in these cells should be analyzed with this in mind. Similar numbers of wt, and TIA-1^{-/-} MEF cells were infected with SFV at MOI 10. Total protein production in the infected cells was analyzed at different times postinfection. Complete infection was determined by immunofluorescence. High MOI infection of the wt B6 MEFs resulted in very efficient shutoff (Figure 4A). It can be seen that host protein production was undetectable after 2.5 h of infection. Viral proteins, on the other hand, were first detected at this time and were efficiently expressed thereafter. In contrast, the TIA-1^{-/-} B6 cells were capable of cellular protein production at much later times than the wt cells. For example, actin was clearly expressed at all times, indicating that shutoff was significantly delayed in these cells. As with the eIF2 α A/A mutant MEFs (Figure 1A), viral protein production did not seem to be affected by the mutation. Similar results were observed with TIAR^{-/-} cells (our unpublished data).

Because it has recently been shown that the TIA-1^{-/-} cells are impaired for SG assembly (Gilks *et al.*, 2004), we determined the effect on SFV-induced SG formation by the TIA-1 deletion. Using immunofluorescence with the 3E6 antibody, we observed an overall reduction in SG numbers in TIA-1^{-/-} relative to wt cells (Figure 4B), even though the time of appearance and disappearance did not differ. These SGs were probably formed by TIAR-mediated aggregation of the 48S* complexes. Figure 4C shows that the phosphorylation of eIF2 α occurred in SFV-infected TIA-1^{-/-} cells.

One possibility for the slower kinetics of shutoff in the TIA-1^{-/-} cells is that there is a lower level of 26S mRNA available for competition with the cellular mRNA transcripts. Both TIA-1/R proteins are RNA binding proteins and TIAR has been shown to be important for West Nile virus RNA replication (Li *et al.*, 2002). We have observed that at the time points at which the differential shutoff rates were detected, no major difference in the quantities of 42S genomic and 26S subgenomic RNA existed in wt and TIA-1^{-/-} cells (Figure 4D).

SG Disassembly in SFV-infected Cells Correlates with Increasing vRNA Levels

Because SGs are sites for aggregation of unused translational components, including mRNAs (Kedersha *et al.*, 1999), we analyzed the localization of both TIA-1 and vRNA in in-

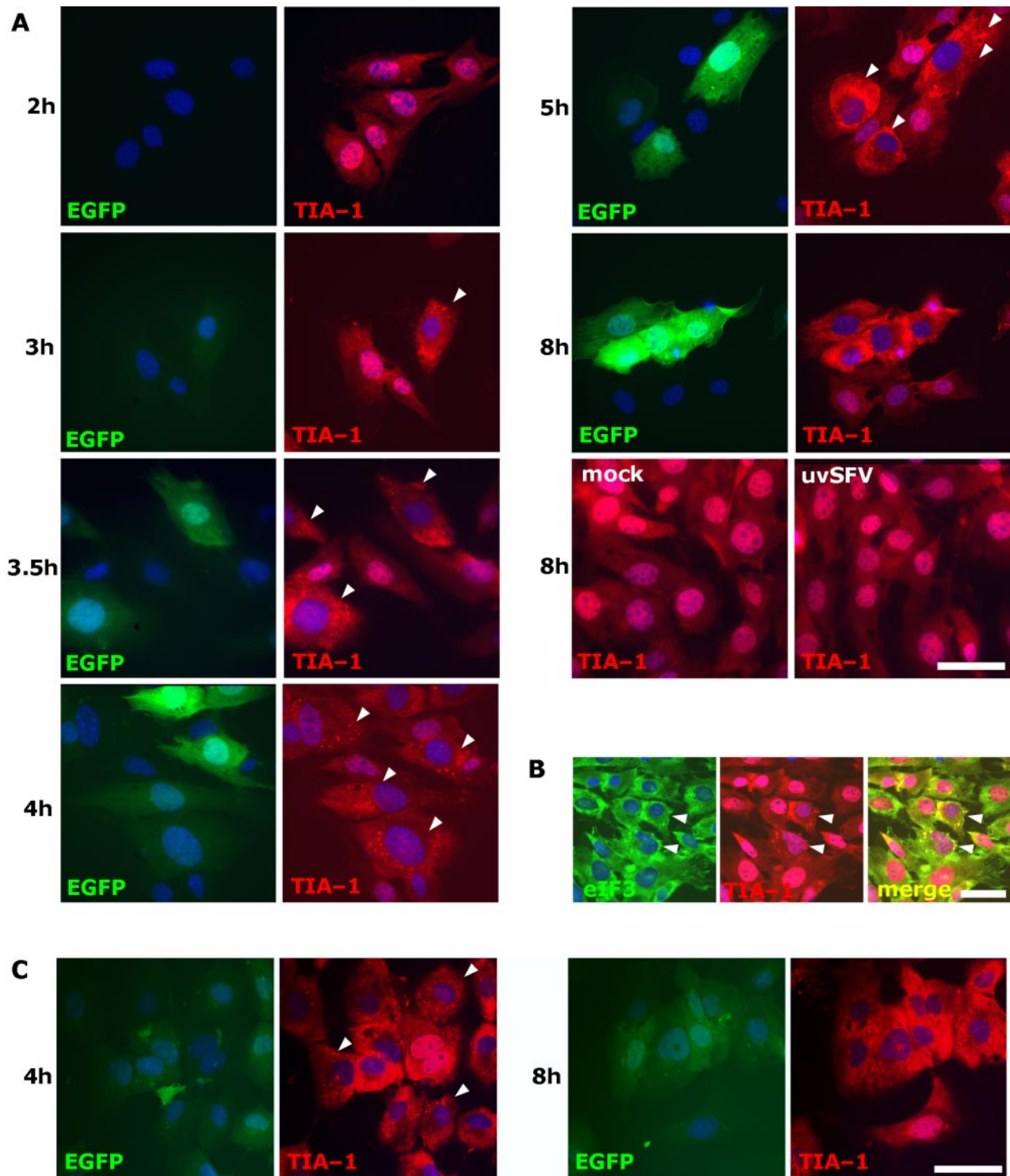


Figure 3. Time course of SG assembly in SFV-infected MEFs. (A) wt MEFs were either infected at low MOI with SFV-b7EGFP, mock infected, or infected with UV-inactivated SFV-b7EGFP. They were then fixed at the indicated times postinfection and processed for immunofluorescence using anti TIA-1 according to *Materials and Methods*. Representative images are shown for each time point. (B) eIF3 is a component of SFV-induced SGs. wt MEFs were infected at low MOI with wt SFV, fixed 5 hpi, and stained for eIF3 and TIA-1. (C) wt MEFs were infected at low MOI with SFV1-EGFP, fixed at the indicated times postinfection, and processed for immunofluorescence using anti TIA-1. Representative images are shown for each time point. Cells containing SGs are indicated with arrowheads. Bar, 50 μ m.

fecting cells to determine whether vRNA was localized to SGs. An explanation for the viral translational supremacy could be that the 26S mRNA has a specific mechanism to

avoid SG association (analogous, perhaps, to that of the hsp70 mRNA in heat shock; Kedersha and Anderson, 2002). Alternatively, vRNA may be SG associated but efficiently

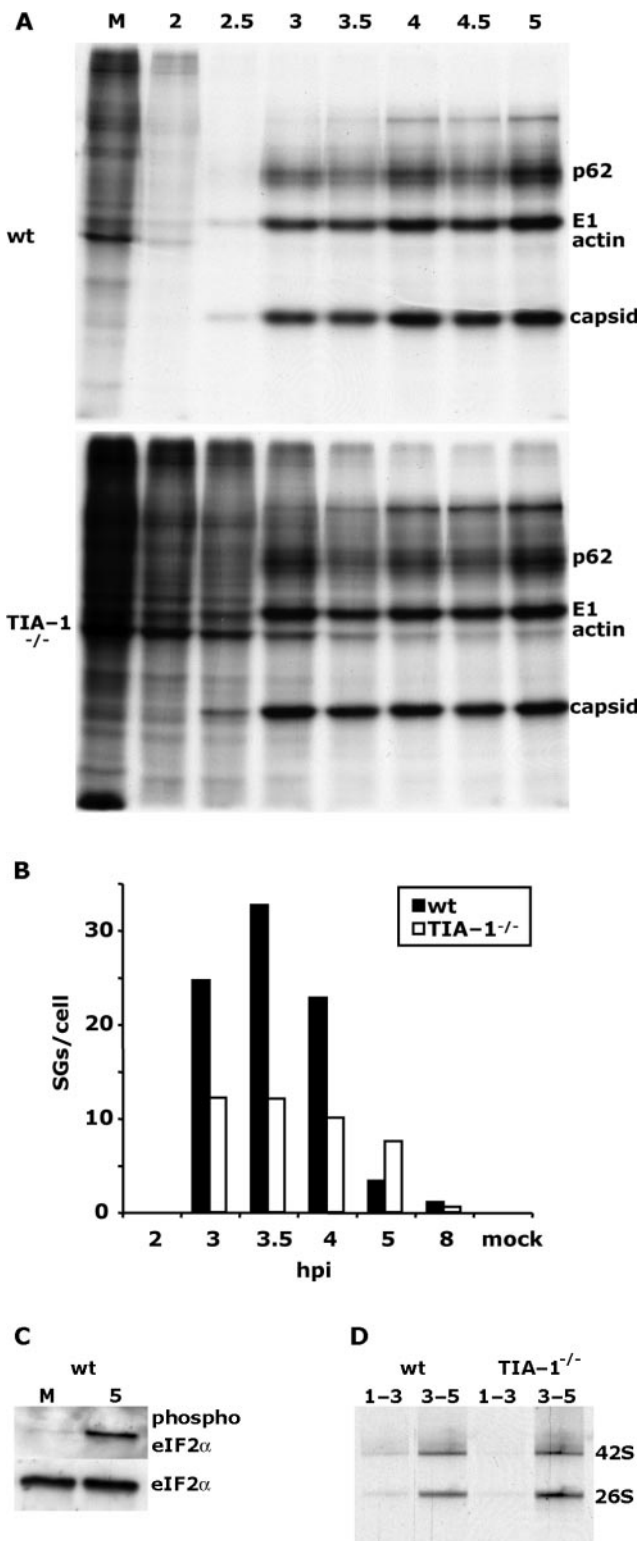


Figure 4. Shutoff is delayed in TIA-1^{-/-} MEFs. (A) Total protein production at various times in wt SFV-infected wt and TIA-1^{-/-} MEF cell lines was analyzed as indicated in *Materials and Methods*. Positions of the cellular protein actin and viral proteins p62, E1, and capsid are indicated. (B) The number of SGs was quantified in wt and TIA-1^{-/-} MEFs infected with SFV-b7EGFP at low MOI. The SGs in 20 cells per time point were counted, and the mean values are plotted. (C) The phosphorylation state of eIF2 α in the TIA-1^{-/-} cells 5 h postinfection or mock infection was determined by Western

translated anyway due to its high level. We determined the relationship between vRNA and SGs by combining ISH analysis for total vRNA and immunofluorescence for TIA-1 protein. wt MEFs were infected at MOI 1 with wt SFV and fixed and processed for ISH. In all cells where a strong vRNA signal was detected, the complete dissolution of SGs was seen, although cytoplasmic localization of TIA-1 was maintained (Figure 5A). Interestingly, we observed that cells at earlier stages of infection showed weaker, more restricted vRNA signals, and generally only contained SGs in areas of little or no vRNA. Uninfected cells within the field again served as internal controls for the specificity of ISH staining. Mock-infected cells gave undetectable vRNA signals (our unpublished data).

We also determined the relationship between vRNA accumulation and SGs using BrUTP to label vRNA produced during 1-h labeling periods only. To specifically label vRNA, ActD was added to the growth medium 30 min before and during each labeling period. A representative time-course experiment is shown in Figure 5B. The results show that SGs assembled before vRNA was detectable by BrUTP staining. As the infection progressed, however, it was evident that the vRNA was not located in SGs. In agreement with the results in Figure 5A, it was observed that the establishment of vRNA replication seemed to occur only in areas where SGs were disassembled. TIA-1 protein was found in a more diffuse pattern in the vicinity of newly produced vRNA. Together, these results show that SFV-induced SGs are dissolved in the vicinity of vRNA as replication progresses.

At Late Times Postinfection, SFV-infected Cells Are No Longer Capable of SG Formation

SGs have been shown to be dissolved when stress inducers are removed and normal protein production is resumed (Kedersha *et al.*, 1999). It is possible, therefore, that the dissolution of SGs at the later stages of SFV infection could reflect the fact that the stress signal only exists early in the infection. Alternatively the SGs may be disassembled even though the stress is still present. To distinguish between these possibilities, we conducted an experiment in which wt MEFs were infected at low MOI with SFV-b7EGFP and subjected to sodium arsenite stress at a late stage. The ability to generate a stress response to the new stress was then analyzed in infected and uninfected cells simultaneously. Figure 6A shows infected cells at 8 hpi that, as expected, exhibited cytoplasmic localization of TIA-1 but no SGs. Figure 6B shows similarly infected cells that were further stressed with sodium arsenite for 30 min before fixation. In this case, only uninfected cells showed SG assembly in response to the new stress. The infected cells displayed diffuse cytoplasmic TIA-1 staining, characteristic of the late stage of SFV infection (Figures 3 and 5). This result showed that SFV-infected cells lose their ability to respond to stress by assembling SGs. This suggested that the SG disassembly seen in the infected cells resulted from this stress resistance rather than the removal of stress signals.

blotting (top) and related to total eIF2 α levels by stripping and reprobing the membrane (bottom). (D) vRNA production at the indicated times during infection of wt and TIA-1^{-/-} MEF cell lines was analyzed as indicated in *Materials and Methods*. Positions of the viral genomic 42S and subgenomic 26S RNAs are indicated. All lanes are labeled in hpi or as M, mock-infected cells.

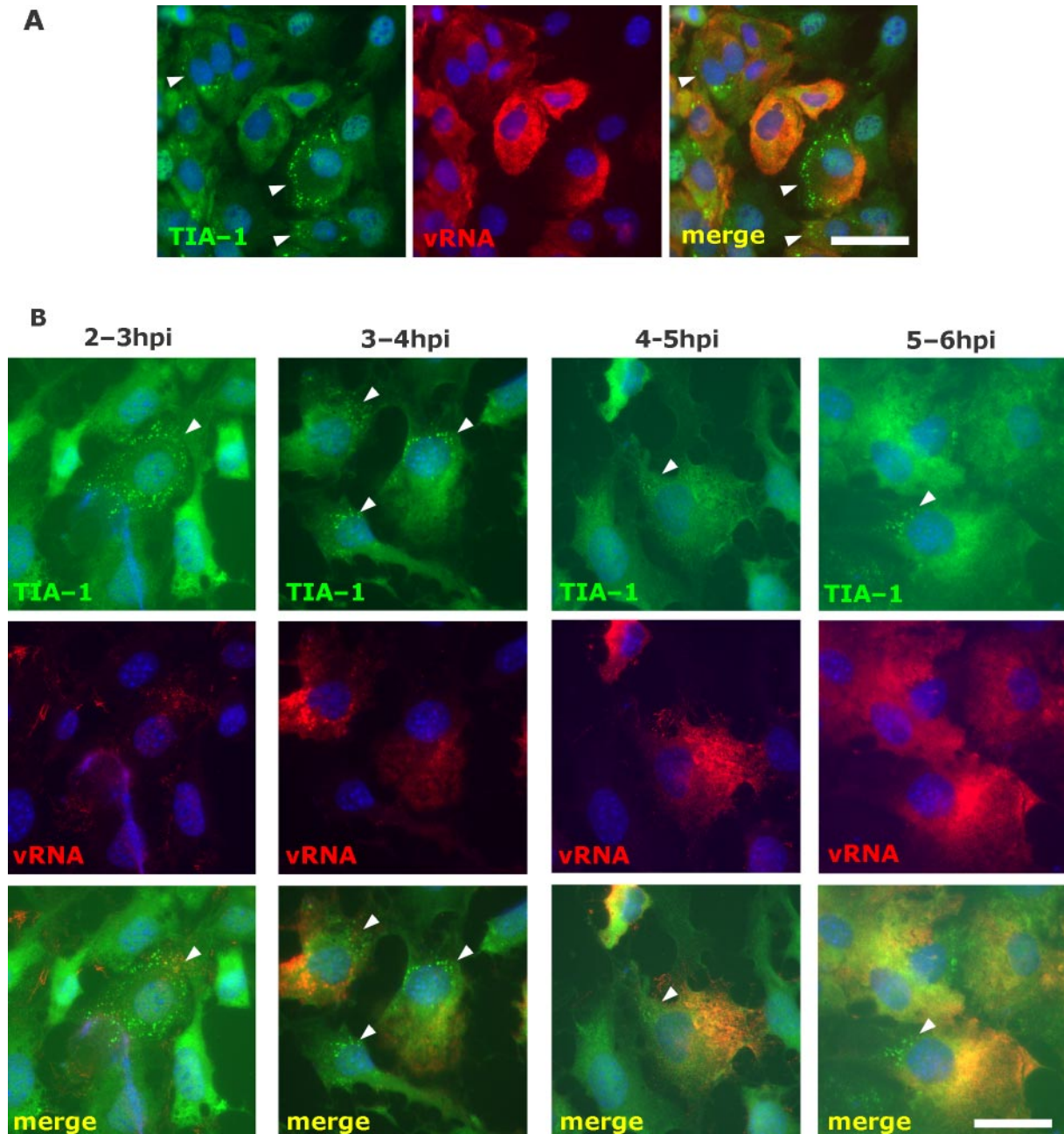


Figure 5. SGs in SFV-infected cells are disassembled as vRNA levels increase. (A) ISH analysis of total vRNA at 5 hpi. wt MEFs were infected at low MOI with wt SFV and fixed at 5 hpi. Hybridization and immunostaining was performed as indicated in *Materials and Methods*. (B) Time course of vRNA accumulation. wt MEFs were infected at low MOI with wt SFV. vRNA produced during the indicated intervals was labeled with BrUTP as indicated in *Materials and Methods* and processed for immunofluorescence using anti TIA-1 and anti BrUTP. Cells containing SGs are indicated with arrowheads. Bar, 50 μ m.

DISCUSSION

The inhibition of translation during viral infection is a well known phenomenon (Gale *et al.*, 2000; Pe'ery and Mathews, 2000). Many mechanisms have been proposed by which viruses both inactivate components of the translation initiation complex and counteract the cell's own infection-induced translation inhibition pathways. In this work, we have investigated the cellular response to infection with SFV. We found that upon infection, host cell translation shutoff is achieved in large part via the phosphorylation of

eIF2 α on serine 51. In other situations, this event leads to a general translation inhibition as discussed above. However, the viral mRNAs have a unique element, the translation enhancer, which we show, confers resistance to these conditions. We also showed that early in the infection, TIA-1/R proteins are mobilized from the nucleus into cytoplasmic foci described previously as SGs, part of the cellular stress response. SGs have previously been shown to be involved in translation regulation under stress via the aggregation of 48S* mRNP complexes. We also show that later in the infec-

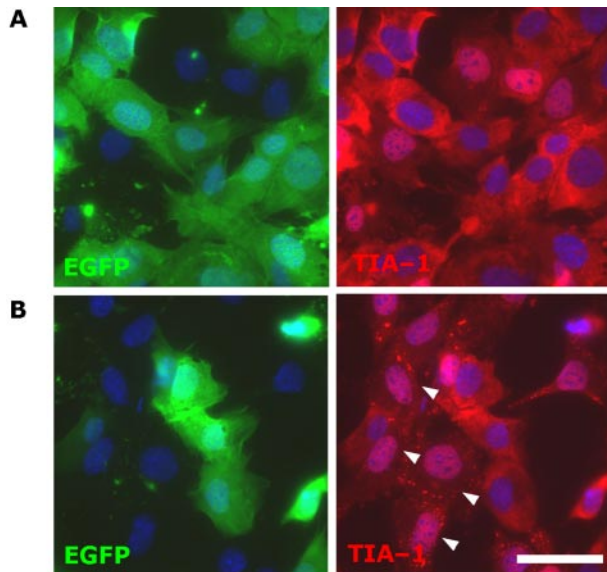


Figure 6. At late times postinfection, SFV-infected cells are no longer capable of arsenite-induced SG formation. wt MEFs were infected at low MOI with SFV-b7EGFP. After 8 h, cells were either fixed directly (A) or treated with sodium arsenite for 30 min and then fixed (B). Cells were processed for immunofluorescence using anti TIA-1. Cells containing SGs are indicated with arrowheads. Bar, 50 μ m.

tion, the SGs are disassembled and TIA-1/R remain cytoplasmic in location. The cells remain unable to assemble SGs in response to another stress.

The initial events after viral entry are uncoating and the initiation of translation of the replicase coding region carried on the 5' two-thirds of the 42S genome. After the switch from translation to replication, the replicase complex catalyzes the production of a complementary copy of the genome (negative strand). From this negative strand, new positive-strand molecules are made (both 42S genomic and 26S subgenomic RNAs). Genome replication of many viruses has been shown to activate PKR (Gale *et al.*, 2000; Barber, 2001; Schneider and Mohr, 2003). A well studied substrate for the kinase activity of PKR is eIF2 α . Some viruses have mechanisms to inhibit the reduction of ternary complex levels by phosphorylation of eIF2 α (Gale *et al.*, 2000; Schneider and Mohr, 2003). These include PKR inhibitors (adenovirus VA RNA [Reichel *et al.*, 1985], vaccinia virus E3L and K3L proteins [Beattie *et al.*, 1991; Chang *et al.*, 1992], influenza virus NS1 protein [Lu *et al.*, 1995], and the herpes simplex virus [HSV] Us11 protein [Mulvey *et al.*, 1999, among others], and eIF2 α phosphatase activators [HSV γ 34.5 protein; He *et al.*, 1997]). Because we have shown that eIF2 α phosphorylation is important for shutoff (Figure 1), we conclude that SFV does not have an eIF2 α kinase inhibitory activity. Rather, SFV seems to tolerate eIF2 α phosphorylation due to the presence of the enhancer element that we have shown functions well under these conditions (Figure 2). Although the enhancer has previously been shown to function only in infected cells (Frolov and Schlesinger, 1994b; Sjöberg and Garoff, 1996), we have extended this to include cells under arsenite stress. Although the translational enhancement in this situation was less than that seen in infected cells, the enhancer significantly increased protein production during stress. This suggests that this element, present in other alphaviruses (Frolov and Schlesinger,

1994b), represents an evolutionary adaptation to the translational block imposed by PKR-mediated eIF2 α phosphorylation. Recently, a novel internal ribosome entry site (IRES) was identified in the cricket paralysis virus (CrPV) genome that can initiate translation independently of the ternary complex (Wilson *et al.*, 2000) and thus is not subject to the profound translation inhibition caused by activation of PKR. The translation of the capped SFV 26S mRNA is unlikely to be driven by a mechanism similar to that of the ternary complex-independent CrPV IRES. It has a very short 5'-untranslated region that is unlikely to contain an analogous RNA structural element. Furthermore, methionine is conserved as the N-terminal amino acid in all alphavirus capsid proteins that would be unnecessary if translation was ternary complex independent. Despite the obvious differences, the CrPV IRES and the alphavirus translational enhancer are both present to facilitate the efficient initiation of translation of the viral structural protein coding regions under conditions where normal cellular mRNAs are severely inhibited due to reduced availability of ternary complexes.

In the absence of ternary complexes under stress, stalled preinitiation complexes (48S*) are assembled (Kedersha *et al.*, 2002). These complexes contain all components of the normal 48S complex except eIF2 and eIF5. The 48S* complexes are then incorporated into SGs by the TIA-1/R proteins. Because the SG components are in dynamic equilibrium with polysomes (Kedersha *et al.*, 2000), constant monitoring is maintained for stress recovery. When charged ternary complexes become available, translation competent initiation complexes form on mRNAs, and the equilibrium will shift toward polysomes. The assembly and disassembly of SGs seems to be mediated by TIA-1/R (Kedersha *et al.*, 1999). We have observed the transient appearance of SGs in SFV-infected cells (Figures 3 and 5). SGs occur in cells at a time when cellular translation is inhibited, but vRNA replication and viral structural protein production are not yet fully efficient. Later, as vRNA replication develops, the SGs are disassembled (Figure 5). It seems that SGs, initially distributed throughout the cytoplasm, become limited to regions away from sites of vRNA replication. Eventually, vRNA is distributed throughout the cell, and the SGs are no longer evident. Although SGs are disassembled in regions containing a high concentration of newly produced vRNA, the TIA-1 staining is not lost in these areas but seems evenly distributed. Interestingly, this occurs despite the elevated levels of phospho-eIF2 α in the cells at these times (Figure 1). Furthermore, we showed that the cells, at these time points, have lost the ability to form SGs in response to arsenite (Figure 6). Due to the dynamic equilibrium between polysomes and SGs, the latter's disassembly has the consequence that the components are again made available for assembly into translation-active polysomes. Thus, we speculate that this occurs to give the 26S mRNA (by these times, present in increased numbers) access to the translation machinery. Cellular mRNAs will remain inhibited for translation due to the eIF2 α phosphorylation, by which, we have shown, the enhancer-containing viral 26S mRNA is less affected (Figure 2).

A question raised by this work concerns the fate of the SG-associated cellular mRNAs. Why does cellular translation not resume after SG disassembly? On one hand, these mRNAs would not be expected to be translated efficiently due to the fact that eIF2 α remains phosphorylated and ternary complex availability is low. Also, it is very possible that these mRNAs are selectively degraded in the SGs or in other cytoplasmic mRNA processing bodies. Furthermore, in alphavirus-infected cells there is a significant decrease in cellular mRNA and rRNA transcription (Frolova *et al.*, 2002;

our unpublished observations). This inhibition, which follows the translational inhibition, also would serve to reduce the cytoplasmic pools of cellular mRNAs that are available for translation and thus consolidate the shutoff at later times.

The delay in host translation inhibition in the TIA-1^{-/-} MEFs (Figure 4) suggests an important role for SGs in the shutoff process at these early times postinfection. At times when viral structural protein synthesis is beginning, the TIA-1^{-/-} cells are still efficiently producing cellular proteins. We have shown in Figure 3 that SGs are prevalent in infected wt cells at these times. Because there is a reduced ability to form SGs in the TIA-1^{-/-} cells (Figure 4B; Gilks *et al.*, 2004), we propose that in these cells, the cellular mRNAs are less efficiently removed from active polysomes and can now compete with the low initial levels of newly produced 26S mRNA for active initiation complexes. As replication develops, increasing quantities of viral transcripts (containing the enhancer) inevitably outcompete the cellular mRNAs for access to initiation complexes and translational advantage is gained. SG assembly by the TIA-1/R proteins is well described, but other cellular proteins have been shown to be involved. Recently G3BP, an endoribonuclease, has been shown to initiate SG formation in a phosphorylation-dependent manner (Tourrière *et al.*, 2003). Also, the Fragile X Mental Retardation protein has been shown to form cytoplasmic mRNA and protein aggregates reminiscent of SGs (Mazroui *et al.*, 2002). Whether these or other unidentified proteins play a role in SFV-induced SGs in wt or TIA-1^{-/-} MEFs is not known.

Other activities of TIA-1/R have recently been described to be involved in various aspects of the life cycles of other viruses. Both TIA-1/R have been shown to interact with West Nile virus (WNV) RNA, and this interaction seems to be necessary for RNA replication (Li *et al.*, 2002). Similarly, Sendai virus (SeV) RNA replication generates short transcripts that bind TIAR and modulate virus-induced apoptosis (Iseni *et al.*, 2002). Interestingly wt SeV infection gave rise to SG formation in a small fraction of cells that was increased when a viral mutant was used containing a deletion in the TIAR binding site. Because neither WNV nor SeV induces efficient shutoff in infected cells, it may well be a common mechanism between these diverse viruses to bind TIA-1/R in the cytoplasm to avoid SG formation and consequent translational repression and perhaps apoptosis. Recently, it has been described that TIA-1/R is mobilized from the nucleus to the cytoplasm in HSV-1-infected cells (Esclatine *et al.*, 2004), and a mutant lacking the virion shutoff protein gene U_L41 led to assembly of SGs.

In conclusion, we have studied the role of the cellular stress response in the regulation of translation during SFV infection. Although there may be distinct but cooperating mechanisms for translational shutoff to be achieved in alphavirus-infected cells (as suggested by other investigators; Strauss and Strauss, 1994; Gorchakov *et al.*, 2004), our results describe a major role for the phosphorylation of eIF2 α in the inhibition of cellular gene expression during SFV infection. The enhancer containing 26S mRNA, however, is clearly less dependent on normal levels of the GTP-eIF2-tRNAi^{Met} ternary complex and consequently SFV- and SFV-based vectors encoding this element, are capable of high levels of protein production under these conditions. We also have shown that SGs play a role, at least in the early stages, in inhibiting the translation of cellular mRNAs. We propose that the disassembly of SGs that occurs later in infection allows the viral 26S mRNA to avoid inclusion in these structures and thereby aids the establishment of translational superiority.

ACKNOWLEDGMENTS

We thank Graham Belsham, Åsa Hidmark, and Gunilla Karlsson for stimulating discussions and critical reading of the manuscript, and Eivor Bonin, Henrik Claesson, and Håkan Samuelsson for technical assistance. This work was supported by the Swedish Research Council and National Institutes of Health Grants AI-50167 and AI-33600.

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